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Characteristics of *Streptococcus suis* strains isolated from 2014 to 2018 in the Swiss pig population linked to the project "PathoPig"

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1 Abstract

The “PathoPig” project was launched in 2014 by the Federal Food Safety and Veterinary Office (BLV) with the aim to re-increase porcine necropsies and subsequently improve the health in swineherds. In the present study, “PathoPig” cases with isolation of *Streptococcus suis* in the time period 2014 to 2018 were further evaluated. For each case, the clinical symptoms on herd basis were assessed. Moreover, the 45 *Streptococcus suis* strains isolated from 43 diseased pigs from 37 independent Swiss farms were further characterized by serotyping and antimicrobial resistance testing. The most commonly reported clinical symptom was sudden death and the most common serotype was serotype 9 (n = 10, 22.2%), followed by serotype 6 (n = 8, 17.8%). All tested isolates were susceptible to penicillin G, ampicillin, ceftiofur, florfenicol and enrofloxacin. 36 isolates (80.0%) were resistant to tetracycline.

Keywords: clinical strains, pigs, resistance profiles, serotypes, *Streptococcus (S.) suis*

2 Zusammenfassung

Das Projekt “PathoPig” wurde 2014 vom Bundesamt für Lebensmittelsicherheit und Veterinärwesen (BLV) ins Leben gerufen, um die Anzahl an Schweinesektionen zu erhöhen und damit die Gesundheit auf Herdenebene zu verbessern. In dieser Studie wurden „PathoPig“ Fälle aus den Jahren 2014 bis 2018 untersucht, bei denen *Streptococcus suis* isoliert wurde. Für jeden „PathoPig“ Fall wurden die klinischen Symptome auf Herdenbasis beurteilt. Ausserdem wurden die 45 *Streptococcus suis* Stämme, isoliert aus 43 erkrankten Schweinen von 37 unabhängigen Schweizer Betrieben, mittels Serotypisierung und antimikrobieller Resistenzprüfung untersucht. Die am häufigsten berichtete Anamnese war plötzlicher Tod und der häufigste Serotyp war Serotyp 9 (n = 10, 22.2%), gefolgt von Serotyp 6 (n = 8, 17.8%). Alle getesteten Isolate waren empfindlich gegenüber Penicillin G, Ampicillin, Ceftiofur, Florfenicol und Enrofloxacin. 36 Isolate (80.0%) waren resistent gegenüber Tetrazyklin.

Schlüsselwörter: Klinische Stämme, Schwein, Resistenzprofil, Serotypen, *Streptococcus (S.) suis*

3 Introduction

Streptococcus (S.) suis is a Gram-positive, facultative anaerobic bacterium responsible for major economic losses in the swine industry due to septicaemia, meningitis, endocarditis, arthritis and also pneumonia (Dutkiewicz et al., 2017; Wisselink et al., 2000). This bacterium colonizes naturally the upper respiratory tract of pigs from all ages, but mainly causes disease in weaned piglets (Dutkiewicz et al., 2017). *S. suis* is also considered a relevant zoonotic agent, especially for people with occupational exposure to swine (Dutkiewicz et al., 2017; Dutkiewicz et al., 2018; Xia et al., 2018). Molecular serotyping is a widely used method for characterization of the isolates and is based on the antigenic diversity of the polysaccharide capsule (CPS) surrounding *S. suis* (Goyette-Desjardins et al., 2014). Currently, there are 29 serotypes described (Kerdsin et al., 2014). Worldwide, serotype 2 is the most common reported serotype to cause infections in pigs, followed by serotype 9 and 3. In humans, the most frequently identified serotypes are serotype 2, followed by serotype 14 (Dutkiewicz et al., 2017).

The "PathoPig" project was launched in 2014 by the Federal Food Safety and Veterinary Office (BLV) with the aim to re-increase porcine necropsies and subsequently improve herd health. To submit pigs as part of the "PathoPig" project, at least one of the following criteria had to be met at farm level: High morbidity and/or mortality, unusual clinical signs, recurrent problems of unknown aetiology resistant to therapy or increased use of antimicrobial drugs. Per case, up to three pigs could be sent in for necropsy (Schediwy et al., 2018).

This study analysed *S. suis* isolates collected by the Section of Veterinary Bacteriology of the Institute for Food Safety and Hygiene from dissected pigs of the "PathoPig" project regarding clinical symptoms, serotypes, causality and resistance profiles in order to assess the occurrence and the impact of *S. suis* in the Swiss pig population.

4 Material and Methods

4.1 Bacterial strains

51 clinical *S. suis* strains isolated from 2014 to 2018 in the course of the project "PathoPig" were available for this study. The strains had been identified by matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS, Bruker, Bremen, Germany) and stored at -80 °C. For each strain, the pathological report with detailed information about the affected animal, the specific anamnestic data and the sampled material was available.

4.2 Culture conditions and DNA extraction

The strains were plated on Columbia blood sheep agar (Thermo Fisher Diagnostics AG, Pratteln, Switzerland) and incubated overnight at 37 °C under aerobic conditions. Grown colonies were verified for purity and if required subcultures under the same growing conditions were performed.

DNA was extracted using a lysis buffer containing 20 ml 1 M TrisHCl (pH 8.5), 100 µl Tween 20, 48 mg Proteinase K and 32 ml Aqua bidestillata. Colony material was dissolved in 400 µl aliquots of this lysis buffer, incubated at 60 °C for 45 min at 850 rpm and subsequently at 96 °C for 15 min at 850 rpm (BioShake iQ, Huberlab, Aesch, Switzerland). The supernatant was won after centrifugation at 13.3 rpm for 5 min and the resulting DNA concentration measured with NanoDrop 2000c (Thermo Fisher Scientific, Reinach, Switzerland). All PCR reactions in this study were performed with a DNA concentration between 20 and 30 ng/µl.

4.3 Evaluation of the PCR products

The PCR products were analysed by capillary electrophoresis with QIAxcel Advanced (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The resulting electropherograms were viewed with the software QIAxcel ScreenGel 1.2.0 (Qiagen, Hilden, Germany) and the threshold set at 0.100 relative fluorescence units.

4.4 *S. suis* species confirmation by recN PCR

To confirm the MALDI-TOF MS based species identification, a PCR targeting the recombination/repair protein gene *recN* described by Ishida et al. (2014) was performed with some modifications. The PCR mixture contained 5.0 µl of HotStar Taq Master Mix (Qiagen, Hilden, Germany), 4 µl of DNA and 0.5 µl of primer mix (10 µM), resulting in a final concentration of 0.5 µM per primer. A total reaction volume of 10 µl with following cycling conditions was used: initial activation of the HotStarTaq DNA polymerase at 95 °C for 15 min; 40 cycles at 94 °C for 30 sec (denaturation), at 60 °C for 30 sec (primer annealing) and at 72 °C for 30 sec (primer extension); final extension at 72 °C for 10 min.

4.5 Serotyping by multiplex PCR

Serotyping was performed according to Kerdsin et al. (2014) with some modifications. With this multiplex PCR, all 29 *S. suis* serotypes are detected in a set of four reactions. Due to the similarity of the *cps* locus between serotype 1 and 14 and 2 and 1/2, respectively, these serotypes cannot be differentiated. Therefore, they will be further referred to as 1 or 14 and 2 or 1/2. The PCR mixture contained 5.0 µl of HotStar Taq Master Mix (Qiagen, Hilden, Germany), 4 µl of DNA and 1.0 µl of primer mix (2 µM), resulting in a final concentration of 0.2 µM for each primer in every set. A total reaction volume of 10 µl with following cycling conditions was used: initial activation of the HotStarTaq DNA polymerase at 95 °C for 15 min; 30 cycles at 94 °C for 30 sec

(denaturation), at 62 °C for 60 sec (primer annealing) and at 72 °C for 60 sec (primer extension); final extension at 72 °C for 10 min.

4.6 Antimicrobial resistance testing

The minimal inhibitory concentration (MIC) was determined by broth microdilution in H-broth (Merlin diagnostics GmbH, Germany) using the Micronaut-S livestock 3 susceptibility plate (Merlin diagnostics GmbH, Germany). The isolates were classified as susceptible or resistant according to clinical breakpoints specific for *S. suis* from the Clinical and Laboratory Standards Institute (CLSI) document VET08 (Anonymous, 2018). If no clinical breakpoints were available, MIC50 and MIC90 were calculated. The tested antimicrobial agents were amoxicillin/clavulanic acid, ampicillin, ceftiofur, cephalothin, enrofloxacin, erythromycin, florfenicol, gentamicin, penicillin G, spectinomycin, tetracycline, tiamulin, tilmicosin, trimethoprim/sulfamethoxazole and tulathromycin.

5 Results

5.1 Origin of the isolates

From the 51 available isolates, 45 were confirmed as *S. suis* by the *recN* PCR. These 45 isolates can be attributed to 38 independent "PathoPig" cases (in total 43 pigs) coming from 37 farms. One farm represents two separate cases, as two pigs with an interval of more than a year were submitted for necropsy. 36 of the farms are located in 12 different Swiss cantons, namely Lucerne (n = 10), Aargau and Thurgau (n = 5), Schaffhausen and Zurich (n = 3), Basel Land and St. Gallen (n = 2), Appenzell Innerrhoden and Ausserrhoden, Glarus, Obwald, Schwyz and Ticino (n = 1). One farm is located in the commune of Büsingen, a German enclave in Switzerland.

5.2 Clinical symptoms

The most commonly reported clinical symptom of the 38 "PathoPig" cases was sudden death, followed by neurological symptoms, lameness, respiratory symptoms and wasting (Figure 1).

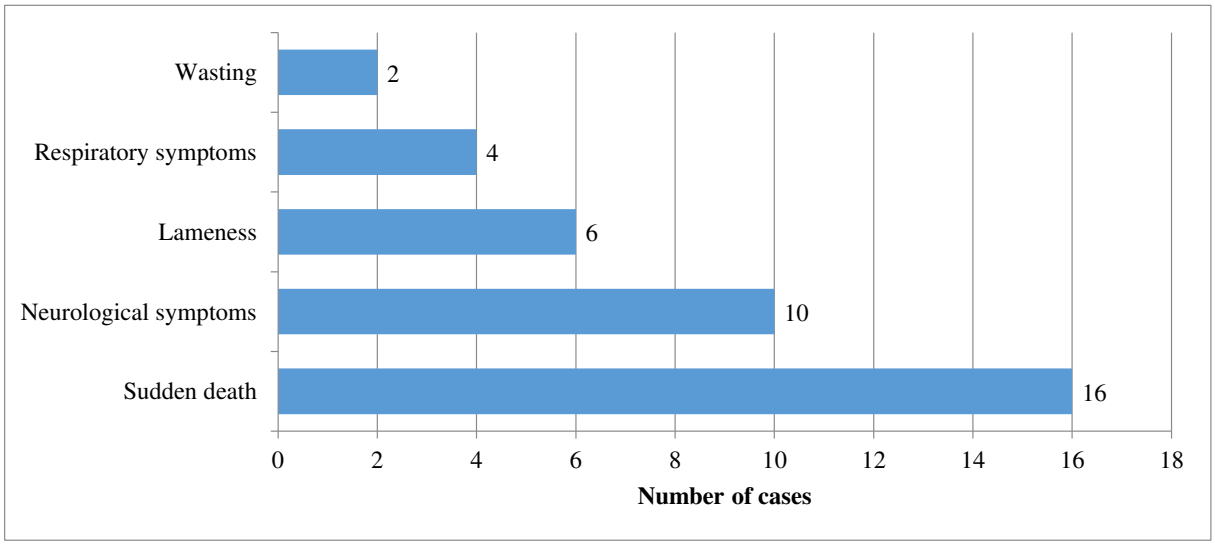

Figure 1: Anamnestic data of the 38 "PathoPig" cases.

Sudden death was reported in all age categories and was the most common clinical manifestation in suckling piglets (n = 9, 69.2%). The most common clinical signs in weaned piglets were neurological symptoms (n = 6, 40.0%), followed by sudden death (n = 4, 26.7%). Lameness was described for every age category except for adults, being the most reported clinical sign in fattening pigs (n = 4, 44.4%).

5.3 Serotypes

Among the 45 *S. suis* isolates, 13 of the currently 29 described serotypes were detected in this study (Figure 2). An overview of the detected serotypes, the sampling material and the age of the affected pigs is shown in Table 1.

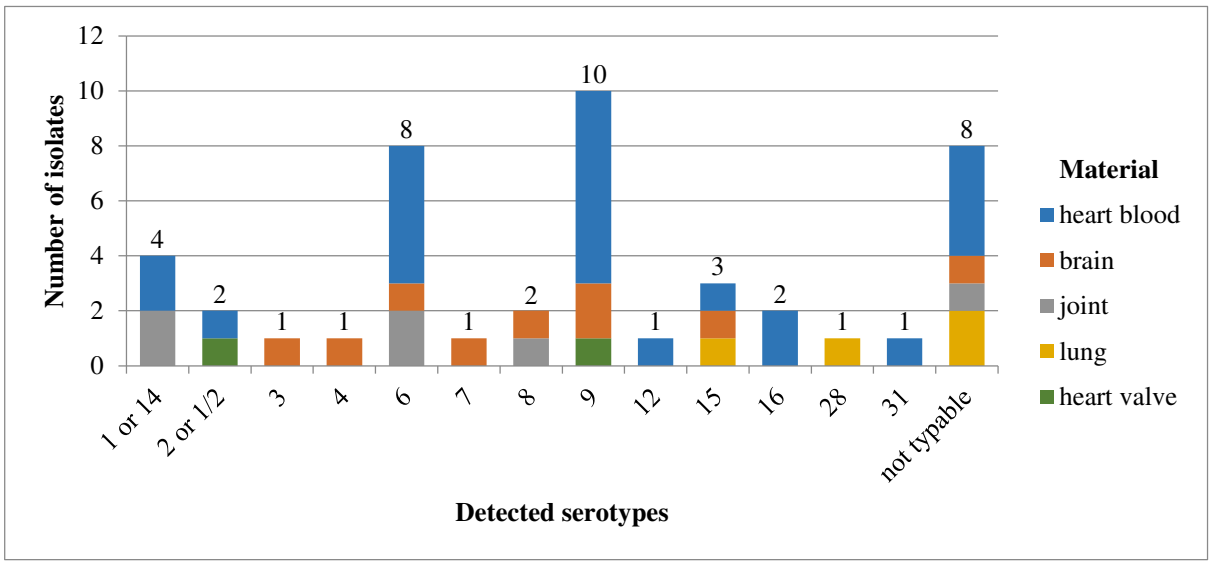

Figure 2: Serotypes of the 45 *S. suis* isolated from the different sampling materials.

The most common serotype was serotype 9 (n = 10, 22.2%), followed by serotype 6 (n = 8, 17.8%). All isolates of the serotype 9 were found in weaned piglets, with heart blood being the most common material of isolation (n = 7, 70.0%). These ten serotype 9 isolates can be attributed to seven farms and nine pigs. One of the isolates of the heart blood and one of the brain were isolated from the same pig. Serotype 6 was exclusively isolated in suckling piglets and the most common material of isolation was heart blood as well (n = 5, 62.5%).

The serotypes 1 or 14 were determined four times, once in a fattening pig in the joint and twice in the heart blood of suckling piglets. In one of these suckling piglets it was simultaneously isolated in the joint. Serotype 15 was detected three times, once in a suckling piglet in the lung, once in a fattening pig in the brain and once in an adult in the heart blood. The serotypes 2 or 1/2 were detected twice, once in a weaned piglet in heart blood and once in the heart valve of a fattening pig. The serotypes 8 and 16 were isolated twice as well: serotype 8 in the joint of a suckling piglet and in the brain tissue of a weaned piglet and serotype 16 in the heart blood of a suckling and a weaned piglet. The serotypes 3, 4, 7, 12, 28 and 31 were only detected in one sample: Serotype 3, 12 and 28 were isolated in fattening pigs (brain, heart blood and lung, respectively). Serotype 4 was isolated in the brain of a suckling piglet and serotype 7 and 31 in weaned piglets (brain and heart blood, respectively).

For eight isolates, the serotype could not be determined (17.8%). These not typable serotypes were found in all age groups and materials, except in adult pigs and the heart valve.

Table 1: Overview of the serotypes, origins and age groups of the 45 *S. suis* isolates.

serotype	**heart blood**				**brain**				**joint**				**lung**				**heart valve**			
	s	w	f	a	s	w	f	a	s	w	f	a	s	w	f	a	s	w	f	a
1 or 14	2	-	-	-	-	-	-	-	1	-	1	-	-	-	-	-	-	-	-	-
2 or 1/2	-	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1	-
3	-	-	-	-	-	-	1	-	-	-	-	-	-	-	-	-	-	-	-	-
4	-	-	-	-	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
6	5	-	-	-	1	-	-	-	2	-	-	-	-	-	-	-	-	-	-	-
7	-	-	-	-	-	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-
8	-	-	-	-	-	1	-	-	1	-	-	-	-	-	-	-	-	-	-	-
9	-	7	-	-	-	2	-	-	-	-	-	-	-	-	-	-	-	1	-	-
12	-	-	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
15	-	-	-	1	-	-	1	-	-	-	-	-	1	-	-	-	-	-	-	-
16	1	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
28	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1	-	-	-	-	-
31	-	1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
nt	1	1	2	-	-	-	1	-	-	-	1	-	-	2	-	-	-	-	-	-

s: suckling piglets / w: weaned piglets / f: fattening pigs / a: adults / nt: not typable

5.4 Causality of *S. suis* isolation and the pathological lesions

Combining *S. suis* detection and the results of necropsy, the *S. suis* infection was causative for the pathological lesions or for the death of the animal in 27 of 43 dissected pigs (62.8%) (Figure 3).

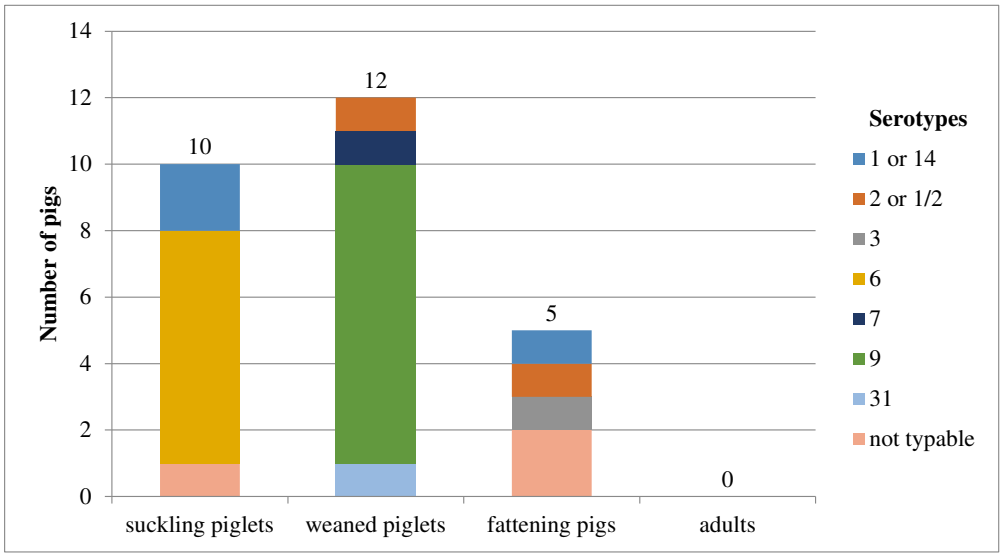

Figure 3: Serotypes that caused with certainty the pathological lesions or the death of the animals.

All isolates belonging to the serotypes 1 or 14, 2 or 1/2, 3, 7, 9 and 31 were considered causative. For serotype 6, seven of the eight isolates were considered causative and for one heart blood isolate the role in the development of the disease was unclear. For all isolates of serotypes 4 and 15 a causality could not be clarified. The isolates belonging to serotypes 12, 16 and 28 were considered contaminants. For one isolate of serotype 8 the role in the lesion development was unclear (joint) and the other was considered a contaminant (brain). From the eight not typable isolates, three were considered causative for the lesions (all from heart blood), whereas for two isolates (heart blood and brain) the role in the development of the lesions could not be clarified. The remaining not typable isolates were considered contaminants.

5.5 Resistance profiles

An overview of the MIC distributions of all isolates is given in Figure 4.

Specific clinical breakpoints for *S. suis* from swine are available for ampicillin, ceftiofur, enrofloxacin, florfenicol, penicillin G and tetracycline. All tested isolates were susceptible to these antimicrobial agents, except to tetracycline. Here, 36 isolates (80.0%) were resistant, thereof all isolates from serotype 9 (n = 10), serotype 8 (n = 2), serotype 15 (n = 3) and the majority of the serotype 6 (n = 6 out of 8) and the not typable isolates (n = 6 out of 8). For erythromycin, trimethoprim/sulfamethoxazole and tulathromycin the tested isolates were divided into two clusters: one cluster with very low MIC values and the other with very high values. 17 isolates showed for all of these three antimicrobial agents low MIC values whereas 9 isolates showed high values. All serotype 1 or 14 isolates and all serotype 6 isolates, except for one, showed low MIC values for all of these three antimicrobial agents whereas the majority of serotype 9 isolates showed high MIC values. All isolates with high values for erythromycin showed at the same time high values for tulathromycin. All *S. suis* isolates showed very low MIC values to beta-lactams antibiotics.

Antimicrobial agent	*S. suis* (n=45)																			
	MIC values (µg/ml)															MIC 50 (µg/ml)	MIC 90 (µg/ml)	Isolates in %		
	0.00781	0.01563	0.03125	0.0625	0.125	0.25	0.5	1	2	4	8	16	32	64	128			S	I	R
Amoxicillin/ clavulanic acid (2/1)[1]								45								≤2	≤2			
Ampicillin				45												≤0.125	≤0.125	100	0	0
Ceftiofur				45												≤0.125	≤0.125	100	0	0
Cephalothin							45									≤1	≤1			
Enrofloxacin					1	26	18									0.25	0.5	100	0	0
Erythromycin				22		2			1		20					0.25	>4			
Florfenicol							22		23							2	2	100	0	0
Gentamicin						1		5	12	12	15					4	8			
Penicillin G			43		2											≤0.0625	≤0.0625	100	0	0
Spectinomycin												3	14	27	1	64	64			
Tetracycline							6	3	7	1		28				>8	>8	13.3	6.7	80
Tiamulin					9		1	6	9	7	3	10				2	16			
Tilmicosin						4				13	4	1	23			>16	>16			
Trimethoprim/sulfamethoxazole (1/19)[1]					27				4	14						≤0.25	>2			
Tulathromycin							20		1	1	3			20		8	>32			

[1]MIC values are given as the MIC of amoxicillin or trimethoprim.
Numbers indicate the number of isolates with corresponding MIC value. White areas indicate the range of dilutions tested for each antimicrobial agent: Values above or below this range denote MIC values greater than the highest concentration tested and MIC values smaller than or equal to the lowest concentration tested, respectively. Vertical lines indicate clinical breakpoints. When two vertical lines exist, the lower breakpoint indicates susceptibility and the higher breakpoint resistance with an intermediate range in between. Clinical breakpoints are available according to CLSI (VET08, 2018, swine).

Figure 4: Distribution of the MIC values for the tested antimicrobial agents of the 45 *S. suis* isolates.

6 Discussion

The distribution of *S. suis* serotypes not only varies between continents and regions, but also changes over time. Worldwide, the most prevalent serotypes isolated from diseased pigs are serotype 2 (27.9%), 9 (19.4%) and 3 (15.9%), followed by 1/2 and 7 to a lesser extent (Dutkiewicz et al., 2017; Goyette-Desjardins et al., 2014). In Europe, serotype 2 and serotype 9 are the most prevalent depending on the country: In Italy, France, Germany and Denmark serotype 2 is the most commonly isolated serotype (Goyette-Desjardins et al., 2014; Prüfer et al., 2019; Wisselink et al., 2000). Several years ago, the serotypes 1 and 14 were the most prevalent in the United Kingdom, but they have been replaced by serotype 2 (Williamson, 2018; Wisselink et al., 2000). In Belgium, the Netherlands, Spain and now also Switzerland serotype 9 is the most prevalent serotype found in diseased pigs (Goyette-Desjardins et al., 2014; Tarradas et al., 2004; Wisselink et al., 2000).

S. suis has been described to cause septicaemia, meningitis, endocarditis, arthritis and pneumonia in pigs, affecting mostly weaned piglets (Dutkiewicz et al., 2017; Wisselink et al., 2000). The clinical symptoms and the sampling material in this study reflect these forms of diseases, as the most common signs were sudden death (hence septicaemia) and neurological symptoms (hence meningitis). These results are in accordance to the clinical signs reported in the UK by Williamson et al. (2018).

The most surprising finding in this study was the high occurrence of serotype 6, exclusively isolated in samples of suckling piglets. Worldwide, this serotype has only been isolated sporadically in a few countries, namely Brazil, Canada, Chile, China, Denmark and Korea, and overall little is known about its virulence (Aarestrup et al., 1998; Costa et al., 2005; Gottschalk and Lacouture, 2015; Gottschalk et al., 2013; Goyette-Desjardins et al., 2014; Morales et al., 2015; Oh et al., 2017; Wei et al., 2009).

Only Chile has reported a high prevalence of this serotype: 28 out of the 29 collected *S. suis* strains from diseased pigs from 2007 to 2011 coming from seven farms were serotype 6, isolated mainly in the brain or the cerebrospinal fluid. Even though the virulence of these Chilean strains could not be proved by a murine model, the authors proposed enhanced virulence as no concomitant infection was present at the time of isolation (Morales et al., 2015). In Korea, serotype 6 was isolated three times (1.3%) from 240 strains from the year 2009 to 2010 and all of them showed the virulence profile *epf-/mrp-/sly+* (Oh et al., 2017). The only reported serotype 6 isolate in Europe originates from Denmark, isolated in the years 1995 and 1996 from a pig with septicaemia (Aarestrup et al., 1998).

The eight serotype 6 isolates retrieved in this study can be attributed to six farms. Half of these isolates were recovered in pure cultures from the investigated organic tissue and the other half as a mixed culture. Along with *S. suis,* three times *Escherichia (E.) coli* and once *Acinetobacter sp*. were isolated. All isolates, except for one from a mixed culture with *E. coli*, were considered causative for the pathological lesions. Therefore, even though the virulence was not specifically tested in this study, these findings suggest that the detected serotype 6 isolates possess enhanced virulence. It could be nevertheless, that these serotype 6 isolates show lower virulence than other serotypes and therefore were mostly found in suckling piglets or in a mixed culture, needing certain predisposing or synergistic factors to cause disease. In literature, *S. suis* is mostly described as a pathogen affecting pigs after weaning at the age of 4 to 10 weeks (Dutkiewicz et al., 2017) and only in a few studies the specific age of the analysed pigs is given (Williamson, 2018; Wisselink et al., 2000). This circumstance could be a reason why *S. suis* is not specifically looked for in suckling piglets and therefore less detected in general in this age group. In a recent study from the United Kingdom though, in which the age for every

serotype was specified, the most prevalent *S. suis* isolated from suckling piglets were of serotype 1, especially in the third week of life. Other isolated serotypes in suckling piglets were serotypes 2, 3, 7 and 14, but to a lesser extent (Williamson, 2018).

Serotyping is based on the antigenic diversity of the polysaccharide capsule (CPS) of *S. suis* (Dutkiewicz et al., 2017; Goyette-Desjardins et al., 2014). The occurrence of not typable strains is not unusual and can be attributed to two reasons: either these are new, not yet described serotypes or known serotypes that are not or poorly encapsulated (Bonifait et al., 2010; Gottschalk et al., 2013; Goyette-Desjardins et al., 2014). The polysaccharide capsule is generally regarded as one of the most important virulence factors for *S. suis*. Nevertheless, it has been described that the CPS can be contra productive in the invasion of the host cells and the biofilm production and that the coexistence of encapsulated and unencapsulated isolates in the same host might be beneficial for the pathogenesis (Dutkiewicz et al., 2018). To determine whether the not typable isolates in this study were encapsulated or not, further investigations like testing for cell surface hydrophobicity or examination by transmission electron microscopy would need to be conducted (Gottschalk et al., 2013; Prüfer et al., 2019). As these not typable serotypes were isolated from similar material as pathogenic serotypes, their virulence capacity should not be excluded as proposed by Gottschalk et al. (2013).

Another interesting finding in this study were the isolates recovered from the lung. None of these four isolates was retrieved in a pure culture. Besides *S. suis*, *Bordetella bronchiseptica*, *Neisseria* sp., *Staphylococcus hyicus*, *Staphylococcus chromogenes*, *Streptococcus alactolyticus* and *Pasteurella multocida* were isolated. Furthermore, two different serotypes were detected and two isolates were not typable. These findings sustain the assertion that *S. suis* is probably not a primary aetiological agent for pneumonia, even though the number of lung isolates is not representative (Feng et al.,

2014; Prüfer et al., 2019; Wisselink et al., 2000). Prüfer et al. (2019) even suggested that the detected serotypes in the lung in their study might represent carrier isolates, as a large number of not typable isolates were found.

The elevated percentage of isolates resistant to tetracycline and the high susceptibility against β-lactams is consistent with the current literature (Kataoka et al., 2000; Li et al., 2012; O'Dea et al., 2018; Vela et al., 2005; Williamson, 2018). Interestingly, all of the serotype 9 isolates were resistant to tetracycline and the majority showed high MIC values for erythromycin, trimethoprim/sulfamethoxazole and tulathromycin, suggesting very low susceptibility against these three antimicrobial agents. This result is similar to a study from Vela et al. (2005), in which 85.7% of the serotype 9 isolates were resistant against tetracyclines, sulphonamides, macrolides and lincosamides. The serotype 6 isolates were susceptible against all tested antimicrobial drugs, except for tetracycline. Here, six out of eight isolates were resistant and one of these isolates showed high MIC values for erythromycin and tulathromycin, suggesting reduced susceptibility against these two drugs. In the study from Chile, in which 28 serotype 6 isolates were detected, five were tested for susceptibility to ampicillin, ceftiofur, penicillin and trimethoprim/sulfamethoxazole. All isolates were susceptible against these antibiotics, which is in accordance to this study (Morales et al., 2015).

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